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INHIBITION OF HUMAN CYTOMEGALOVIRUS PROTEASE BY ENEDIONE DERIVATIVES OF THIENO[2,3-d]OXAZINONES THROUGH A NOVEL DUAL ACYLATION/ALKYLATION MECHANISM

Ivan L. Pinto*[a], Richard L. Jarvest*[a], Brian Clarke[a], Christine E. Dabrowski[b], Ashley Fenwick[a], Michele M Gorczyca[b], L. John Jennings[a], Patrick Lavery[a], Edmund J Sternberg[b], David G. Tew[a] and Andrew West[a]

SmithKline Beecham Pharmaceuticals,

[a]*New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW, UK*

[b]*Upper Providence, Collegeville Rd, Collegeville, PA USA*

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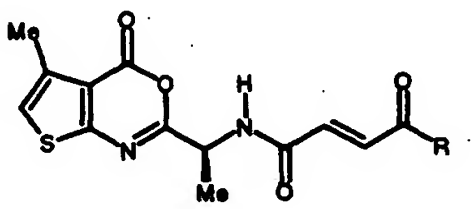
Abstract: Enedione derivatives of thieno[2,3-d]oxazinones are nanomolar inhibitors of CMV protease which act through a novel dual acylation of the catalytic serine and alkylation of the protease cysteine 161 *via* a Michael addition to the enedione moiety of the inhibitor.

The finding that all herpes viruses encode a unique protease that is essential for viral replication has afforded a potential new target for therapeutic intervention.[1] This potential has been further enhanced by the publication of the crystal structure of the human cytomeglovirus protease showing it to be a serine protease with a catalytic triad consisting of His63, His157 and Ser132 which is unique to the herpes proteases.[2] The protease plays a vital role in viral capsid maturation, cleaving a scaffold protein which is encoded in-frame with the C-terminal part of the gene product.[3] The protease shows a varying degree of sequence homology across the herpesvirus family and a highly conserved P4-P1' cleavage motif in which proteolysis occurs between alanine and serine residues.



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There have been several mechanism based inhibitors reported recently all of which interact with the catalytic serine.[4,5] We now report on a series of enedione-thieno[2,3-d]oxazinones derivatives **1** related to those described in an earlier letter[6] which are potent and selective CMV protease inhibitors that act by not only acylating the catalytic serine but also by alkylating cysteine 161 of the protease.

The enedione-thieno[2,3-d]oxazinones **5-16** were prepared from the thiophene amino acid **2** by acylation with Boc-alanine followed by concommitant cyclisation to the thieno[2,3-d]oxazinone **3** using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC). Treatment of **3** with trifluoroacetic acid yielded the amine salt in excellent yield which could be acylated with the appropriate acid using DEC in the presence of Hunigs base (Scheme 1). The enedione-thieno[2,3-d]oxazinones were evaluated as inhibitors in quenched fluorescence assays for the peptidolytic activity of the CMV, HSV-2 and VZV proteases[5] and the results are summerised in Table 1. Surprisingly, an order of magnitude improvement in IC_{50} on CMV, HSV-2 and VZV proteases was observed for the aryl ketones compared to the most potent of the cinnamide thieno[2,3-d]oxazinones described earlier[6] while activity on HSV-2 and VZV was generally micromolar. Although an ethoxy substituent in the 4-position of the aryl ring **6** was detrimental to CMV protease potency, both electron

e-mail: Ivan_Pinto-1@sbphrd.com. Fax +44 1279627841; Richard_L_Jarvest@sbphrd.com. Fax +44 1279627628

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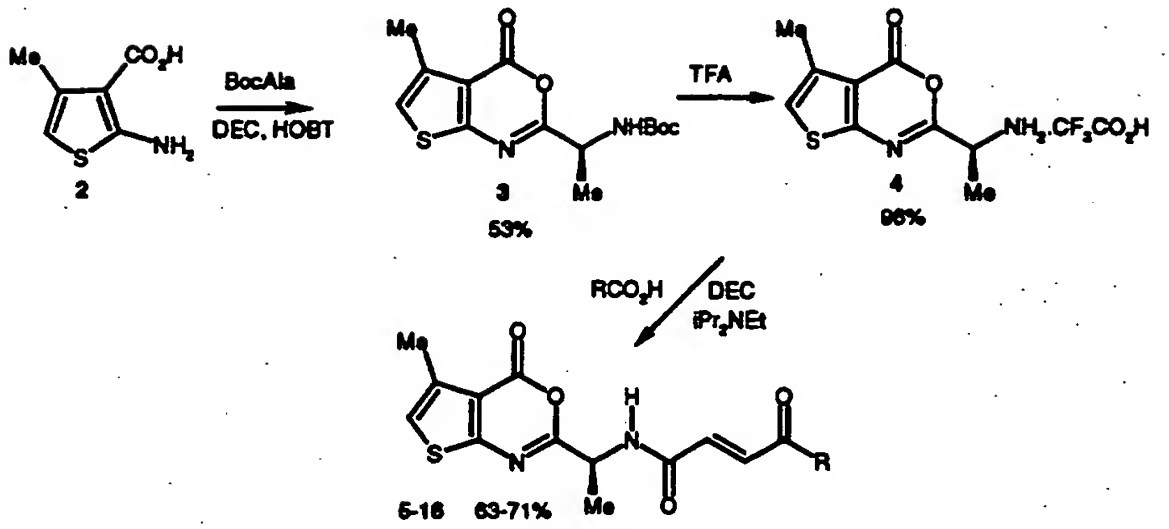
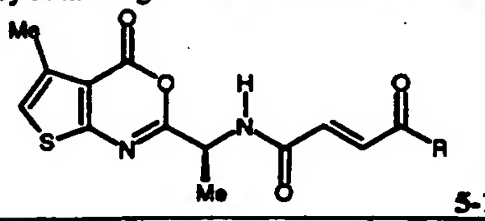


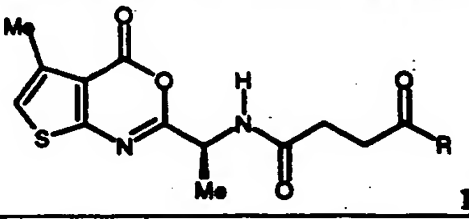
Scheme 1

donating and electron withdrawing substituents were well tolerated in the 3-position (compounds **8** to **10**). When the ketone was replaced by an amide group as in **13** there was a considerable drop in CMV activity though that against the HSV-2 and VZV proteases remained similar to that observed with aryl ketones. Replacement of the phenyl ring of **5** with a methyl group in **14** resulted in at least a ten fold drop in potency, suggesting that the aryl ring remains a key recognition element as in the cinnamide inhibitors. The saturated analogues **15** and **16** were found to have poor activity on the CMV protease, with roughly a 1,000 fold loss of potency as compared to the unsaturated compounds.

The surprisingly good potency on CMV protease with this class of compound led us to speculate that a second inhibitory mechanism may be at play. A Michael type addition onto the enedione function seemed a potential mechanism that was consistant with the key features of the SAR such as the substantial loss of potency in moving from the unsaturated ketone **5** to the unsaturated amide **13** to the saturated ketone **15**.



5-14



15-16

No	R	IC_{50} μM CMV	HSV-2	VZV
5	Ph	0.03	2.7	1.0
6	4-EtO-Ph	0.26	16	1.6
7	4-Cl-Ph	0.06	15	3.2
8	3-MeO-Ph	0.029	0.68	0.34
9	3-CN-Ph	0.041	2.0	0.41
10	3-NO_2-Ph	0.031	3.0	0.48
11	2-NO_2-Ph	0.014	2.2	0.84
12	2-thienyl	0.029	4.6	0.65
13	NHPh	2.3	2.6	1.1
14	Me	0.37	0.79	1.6
15	Ph	21	12	27
16	2-thienyl	19	4.7	0.82

Table 1

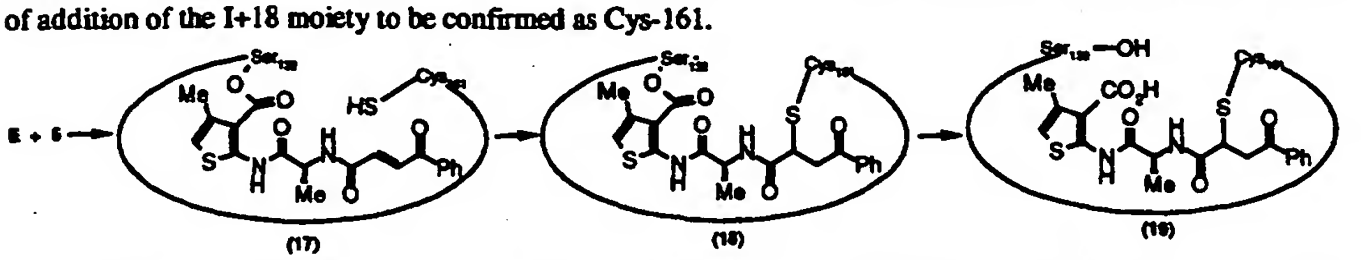
Fig 1

Modelling of **5** as the tetrahedral intermediate with Ser-132 into the active site of CMV protease[2] revealed an excellent fit into the prime-side cleft which resulted in cysteine 161 lying in close proximity (2.6Å) to the amide terminus of the dione double bond, well placed for Michael addition (Fig 1). The Cys-161 residue is not essential for catalytic activity,[7] but in the presence of certain disulphide or redox reagents, the Cys-161 of CMV protease has been shown to form a disulphide bond to Cys-138 which inactivates the enzyme.[8] A similar interaction with the corresponding cysteine in VZV or HSV-2 was not possible from modelling studies due to the enedione function being twisted out of conjugation by helix A6 in these enzymes[2b] precluding an effective fit and thereby conferring selectivity for CMV. Confirmation that the enedione was indeed alkylating Cys-161 was sought from mass spectrum studies of the complex obtained from incubating **5** with CMV protease.

Formation of the inhibitor enzyme complex (I:E) of **5** with CMV protease was carried out at 1:1, 5:1 and 10:1 molar ratios (5:CMV) and analysed using LC/MS with electrospray ionisation (ESI). The results indicated the formation of a complex consistent with the addition of one molecule of **5** to the protease which, on extended incubation, increased in molecular mass by approximately 18 Da. In order to determine the binding site the I:E complex was digested with trypsin (50:1 by weight complex:trypsin) and the tryptic peptides analysed by LC ESI/MS and the data compared with that obtained from a control digest of CMV. An extra tryptic peptide (HVALCSVGR) was observed in the digest of I:E whose monoisotopic molecular mass (1326.6 Da by MALDI ToF; 1326.7 Da theoretical) was consistent with the tryptic peptide containing Cys-161 + **5** + 18 Da, suggesting the hydrolysed form of **5** was bound to this peptide. To confirm this observation the experiment was repeated with the chlorinated analogue **7**. Comparison of the results revealed an extra peptide whose molecular mass (1360.6 Da) was 34 Da higher than that observed with **5**, consistent with the difference in molecular mass of the two ligands. To confirm the nature of the modified peptides the digests were analysed by matrix assisted laser desorption ionisation (MALDI) time of flight (ToF) MS and fast atom bombardment (FAB) MS. Both FAB and MALDI confirmed the molecular mass of the modified peptide. The influence of the chlorine in **7** on the isotope pattern of this peptide established that the compound was indeed bound to this peptide. Post source decay MS/MS analysis, using MALDI ToF, of the modified peptides indicated one major fragment ion equivalent to the loss of a hydrolysed **5** or **7** from the tryptic peptide containing Cys-161.

Studies with thienoxazinones that do not carry a second potentially reactive site have shown that acylation occurs on the active site serine, Ser-132[4e,5] and that subsequent slow deacylation occurs.[5,6] We believe that with the present series of inhibitors, the initial E+I complex observed by MS corresponds to the

schematic structure 18, where initial acylation of the serine has been followed by Michael addition of Cys-161. On extended incubation, the E+I+18 complex results from hydrolysis of the acyl-enzyme bond to afford structure 19. The adduct 19 is stable to further incubation and to the tryptic digest conditions allowing the site of addition of the I+18 moiety to be confirmed as Cys-161.



When representative enediones inhibitors were tested for cytotoxicity in MRC-5 cells using an XTT assay,[5] they were found to be very cytotoxic. The TC_{50} values for 5, 12, and 14 were 14, 2 and 11 μM respectively. The cytotoxicity is presumably related to the Michael acceptor reactivity of the molecules as they were substantially more cytotoxic than other classes of thieno[2,3-d]oxazinone inhibitors.[5,6] The high level of cytotoxicity precluded further evaluation of the enediones inhibitors as CMV antiviral agents.

In conclusion the [2,3-d]thienoxazinone enediones represent the most potent inhibitors of CMV protease to be described to date. The compounds not only acylate the catalytic serine of CMV protease but also alkylate Cys-161 *via* a Michael type addition which probably accounts for the excellent potency observed. It is interesting to note that Tsuge *et al* speculated[2d] that molecules that could covalent bind to Cys-161 would provide inhibitors of CMV protease.

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References and footnotes:

1. a. Liu, F.; Roizman B. *J. Virol.*, **1991**, *65*, 5149. b. Welch, A.R.; Woods, A.S.; McNally, L.M.; Cotter, R.J.; Gibson, W. *Proc. Natl. Acad. Sci. USA*, **1991**, *88*, 10792.
2. a. Tong, L.; Qian, C.; Massariol, M.-J.; Ronneau, P.R.; Cordingley, M.G.; Lagacé, L. *Nature*, **1996**, 383, 272. b. Qiu, X.; Culp, J.S.; DiLella, A.G.; Hellmig, B.; Hoog, S.S.; Janson, C.A.; Smith, W.W.; Abdel-Meguid, S.S. *Nature*, **1996**, 383, 275. c. Shieh H.-J.; Kurumbail, R.G.; Stevens, A.M.; Stegeman, R.A.; Sturman, E.J.; Pak, J.Y.; Wittwer, A.J.; Palmier, M.O.; Wiegand, R.C.; Holwerda, B.C.; Stallings, W.C. *Nature*, **1996**, 383, 279. d. Chen P.; Tsuge, H,; Almassy, R.J.; Gribskov, C.L.; Katoh, S.; Vanderpool, D.L.; Margosiak, S.A.; Pinko, C.; Matthews, D.A.; Kan, C.-C. *Cell*, 1996, **86**, 835.
3. Gao, M.; Matusick-Kumar, L.; Hurlburt, W.; DiTusa S.F.; Newcomb, W.W.; Brown, J.C.; McCann, P.J. III; Deckman I.; Colonno, R.J. *J. Virol.*, **1994**, *68*, 3702.
4. a. Pinto, I.L.; West, A.; Debouck, C.M.; DiLella, A.G.; Gorniak J.G.; O'Donnell, K.C.; O'Shannessy, D.J.; Patel, A.; Jarvest, R.L. *Bioorg. Med. Chem. Lett.*, **1996**, *6*, 2467. b. Jarvest, R.L.; Parratt, M.J.; Debouck, C.M.: Gorniak J.G.; Jennings, L.J.; Serafinowska, H.T.; Strickler, J.E. *Bioorg. Med. Chem. Lett.*, **1996**, *6*, 2463. c. Jarvest, R.L.; Connor, S.C.; Gorniak, J.G.; Jennings, L.J.; Serafinowska, H.T.; West, A. *Bioorg. Med. Chem. Lett.*, **1997**, *7*, 1733. d. Deziel, R.; Malenfant, E., *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 1437. e. Borthwick, A.D.; Weingarten, H.M; Haley, T.M.; Tomaszewski, M.; Wang, W.; Hu, Z.; Bedard, J.; Jin, H; Yuen, L.; Mansour, T.S. *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 365.
5 Dabrowski, C.E.; Ashman, S.M.; Fernandez, A.V.; Gorczyca, M.; Lavery, P.; Parratt, M.J.; Serafinowska, H.T.; Sternberg, E.J.; Tew, D.G.; West,A.; Jarvest, R.L., submitted for publication.
6. Jarvest, R.L.; Pinto, I.L.; Ashman, S.M.; Dabrowski, C.E.; Fernandez, A.V.; Jennings, L.J.; Lavery, P.; Tew, D.G. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 443.
7. Welch, A.R.; McNally, L.M.; Hall, M.; Gibson, W. *J. Virol.*, **1993**, *67*, 7360.
8. a. Baum, E.Z.; Siegel, M.M.; Bebernitz, G.A.; Hulmes, J.D.; Sridharan, L.; Sun, L.; Tabei, K.; Johnston, S.H.; Wildey, M.J.; Nygaard, J.; Jones, T.R.; Gluzman Y. *Biochemistry*, **1996**, *35*, 5838. b. Baum, E.Z.; Ding, W.-D.; Siegel, M.M.; Hulmes, J.; Bebernitz, G.A.; Sridharan, L.; Tabei, K.; Krishnamurthy, G.; Carofiglio, T.; Groves, J.T.; Bloom, J.D.; DiGrandi, M.; Bradley, M.; Ellestad, G.; Seddon, A.P.; Gluzman Y. *Biochemistry*, **1996**, *35*, 5847.